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The E7 protein of the cottontail rabbit papillomavirus immortalizes normal rabbit keratinocytes and reduces pRb levels, while E6 cooperates in immortalization but neither degrades p53 nor binds E6AP

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Abstract

Human papillomaviruses (HPVs) cause cervical cancer and are associated with the development of non-melanoma skin cancer. A suitable animal model for papillomavirus-associated skin carcinogenesis is the infection of domestic rabbits with the cottontail rabbit papillomavirus (CRPV). As the immortalizing activity of CRPV genes in the natural target cells remains unknown, we investigated the properties of CRPV E6 and E7 in rabbit keratinocytes (RK) and their influence on the cell cycle. Interestingly, CRPV E7 immortalized RK after a cellular crisis but showed no such activity in human keratinocytes. Co-expressed CRPV E6 prevented cellular crisis. The HPV16 or CRPV E7 protein reduced rabbit pRb levels thereby causing rabbit $p19^{ARF}$ induction and accumulation of p53 without affecting cellular proliferation. Both CRPV E6 proteins failed to degrade rabbit p53 *in vitro* or to bind E6AP; however, p53 was still inducible by mitomycin C. In summary, CRPV E7 immortalizes rabbit keratinocytes in a species-specific manner and E6 contributes to immortalization without directly affecting p53.

Keywords: P53 protein; Retinoblastoma protein; Human papillomavirus; Cottontail rabbit papillomavirus; E6; E7; Keratinocytes; Immortalization; Rabbit; E6AP

Introduction

The infection with high-risk human papillomaviruses (HPV) is regarded as a necessary risk factor for the development of cervical cancer (Walboomers et al., 1999). For non-melanoma skin cancer (NMSC), which is the most common malignancy in Caucasians worldwide (Miller and Weinstock, 1994; Parker et al., 1996; Stern, 1999), the identification of high-risk papillomavirus types is still under debate although epidemiology and associated risk factors such as age, UV-mediated local and transplantation-related immune suppression suggest the involvement of an infectious agent (Forslund et al., 2007; Iftner et al., 2003). Sufficient evidence for an involvement of specific HPV types, like types 5 and 8, in skin carcinogenesis was found so far only in patients with the rare genetic disorder epidermodysplasia verruciformis (EV) that develop HPV-associated warts, which progress in up to 60% of the individuals into mainly primary squamous cell carcinomas (Jablonska and Majewski, 1994; Pfister, 1992). Skin cancer was also recognized as a common side effect in long-term-immunosuppressed patients as renal transplant recipients, in whom after an extended period of immunosuppressive therapy NMSC can be found in up to 70% (Bouwes Bavinck et al., 1996). HPV-DNA has been detected in more than 80% of malignant skin tumors of immunosuppressed patients (Berkhout et al., 1995; Harwood et al., 2000; Shamanin et al., 1994). Recent studies show that NMSCs of the immunocompetent general population contain HPV-DNA in varying percentages (de Villiers, 1998; Harwood et al., 2000; Iftner et al., 2003). A huge variety of HPV types were detected, including EV-associated, other cutaneous and genital high- and low-risk types (Bouwes Bavinck et al., 2001; Lampert et al.,

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2000). A role of HPV in the development of non-melanoma skin cancer (NMSC), especially squamous cell carcinoma even in the normal population, is therefore very likely.

The underlying mechanisms leading from HPV infection to invasive skin cancers are, however, not yet fully understood. To address this question, we use an animal model to study papillomavirus-induced skin tumors in the domestic rabbit infected with the cottontail rabbit papillomavirus (CRPV) that was discovered by Shope in 1933 (Rous and Beard, 1935; Shope and Hurst, 1933). Infection of rabbits with CRPV particles or viral DNA leads to the development of local tumors within 3 to 6 weeks post-infection. These papillomas progress within 6 to 12 months in more than 80% of the cases into invasive and metastasizing carcinomas without the need of any other known cofactors (Jeckel et al., 2002; Syverton, 1952). Therefore, CRPV provides an excellent in vivo model for studying the malignant progression and immunological mechanisms of PV-associated tumors (Brandsma, 2005; Breitburd et al., 1997). CRPV encodes at least three proteins that confer anchorage independent growth to immortalized cell lines: CRPV E7, which is encoded in open reading frame (ORF) E7, and long (LE6) and short E6 (SE6), encoded in ORF E6 (Meyers et al., 1992). None of the CRPV E6 proteins was previously found to coprecipitate with mouse p53 from embryonal carcinoma cells (Harry and Wettstein, 1996). The CRPV E7 protein shares some of the properties of HPV16 E7, as both bind pRb, disrupt the complex between pRb and the transcription factor E2F, transactivate the adenovirus E2 promotor and mediate cellular transformation of rodent fibroblast lines as measured by growth in soft agar (Defeo-Jones et al., 1993; Haskell et al., 1993; Schmitt et al., 1994). It was shown that nearly every ORF of the CRPV genome except E5 and L2 is required for the development of tumors in the rabbit model (Brandsma et al., 1991, 1992; Meyers et al., 1992; Nasseri et al., 1989). However, up to now CRPV genes have not been demonstrated to be able to immortalize primary cells. Earlier we have already described the binding affinity for the CRPV E7 protein to the tumor suppressor pRb to be of the relative strength of 11% to that of HPV16E7 (Schmitt et al., 1994) and found for CRPV E6/E7 no immortalizing activity in normal human keratinocytes.

The key function of the HPV16 E7 oncoprotein is the activation of cell proliferation in the absence of mitogenic stimuli. This occurs mainly by binding and inactivating pRb family members, which normally inhibit the E2F transcription factor family. Activation of E2F results in cell cycle progression but may also induce $p14^{ARF}$ or its murine homolog $p19^{ARF}$, respectively (Bates et al., 1998; Komori et al., 2005). $p14/p19^{ARF}$ inhibits the ubiquitin ligase activity of mdm2 directed against p53, thus increasing the half-life of the p53 protein (Honda and Yasuda, 1999; Zhang et al., 1998).

E7-induced p53 stabilization should induce apoptosis or growth arrest by the transactivation of proapoptotic genes or p21 respectively. To counteract the antiproliferative and proapoptotic activities, high-risk type E6 proteins recruit the cellular E3-ubiquitin ligase E6AP to force the proteasomal degradation of p53 (Mantovani and Banks, 2001; Scheffner et al., 1990). However, binding and degradation of p53 by E6 proteins appears to be a unique feature of established genital high-risk types and has so far not been demonstrated for E6 proteins derived from low-risk and cutaneous papillomaviruses (Elbel et al., 1997; Hiller et al., 2006; Steger and Pfister, 1992). Therefore, it remains an open question how low-risk genital and cutaneous HPV types escape a p53-mediated growth arrest in spite of the inability of their E6 proteins to directly target the p53 protein.

As the cell culture system to analyze CRPV's *in vitro* properties so far mainly employed already immortalized rodent cells, as NIH3T3 or SfEP1 (Meyers and Wettstein, 1991), we aimed to evaluate the immortalizing properties of CRPV in its natural host cells, normal rabbit keratinocytes. For that reason, we established and characterized rabbit keratinocyte cell lines by retroviral transduction with CRPV E6/E7 and as control HPV16 E6/E7.

Here we show that CRPV E7 on its own was able to immortalize rabbit keratinocytes after a cellular crisis, whereas it had no such activity in normal human keratinocytes. Co-expression of CRPV E6 prevented the cellular crisis of the transduced rabbit keratinocytes. The E7 protein of CRPV and of HPV16 reduced the amount of rabbit pRb in the rabbit keratinocyte lines and caused accumulation of rabbit $p19^{ARF}$, which resulted in stabilization of p53. Nevertheless, this had no negative effect on the cellular proliferation rate. To determine why p53 is stabilized even in the presence of CRPV E6, we investigated the interactions between CRPV E6, p53 and E6AP. The E6 proteins of CRPV and HPV16 failed to bind p53 from cell lysates of rabbit keratinocytes and to degrade rabbit p53 *in vitro*. To clarify the question how CRPV can bypass p53-mediated growth arrest without being capable of degrading p53, we analyzed the cellular localization of the stabilized p53 as well as its expression after DNA damage and proteasomal inhibition. This revealed a mainly nuclear localization and no further stabilization of p53 after proteasomal inhibition. Rabbit p53 was transcriptionally active and still induced p21. In summary, our data provide evidence that E7 of CRPV is sufficient to immortalize keratinocytes in a species-specific mode and that E6 contributes to immortalization but does not influence rabbit p53 and E6AP directly.

Results

CRPV E7 is able to immortalize rabbit keratinocytes by itself and in cooperation with CRPV E6

Normal rabbit keratinocytes (NRK) were infected with recombinant retroviruses. For the combination of CRPV, E6 and E7 simultaneous infection with retroviruses containing CRPV E6 or CRPV E7, respectively, was used. In the case of HPV16 E6/E7, we used retroviruses from the pLXSNHPV16E6/E7 plasmid allowing simultaneous expression of E6 and E7 (Halbert et al., 1991). After infection and drug selection, resistant cell clones were passaged for more than 6 months. The results of the long-term growth analysis of the first 2 months are shown in Fig. 1. The G418-resistant rabbit keratinocyte (RK) lines obtained after infection with viruses containing CRPV E6, HPV16 E6 or the parental vectors pZip or pLXSN alone showed

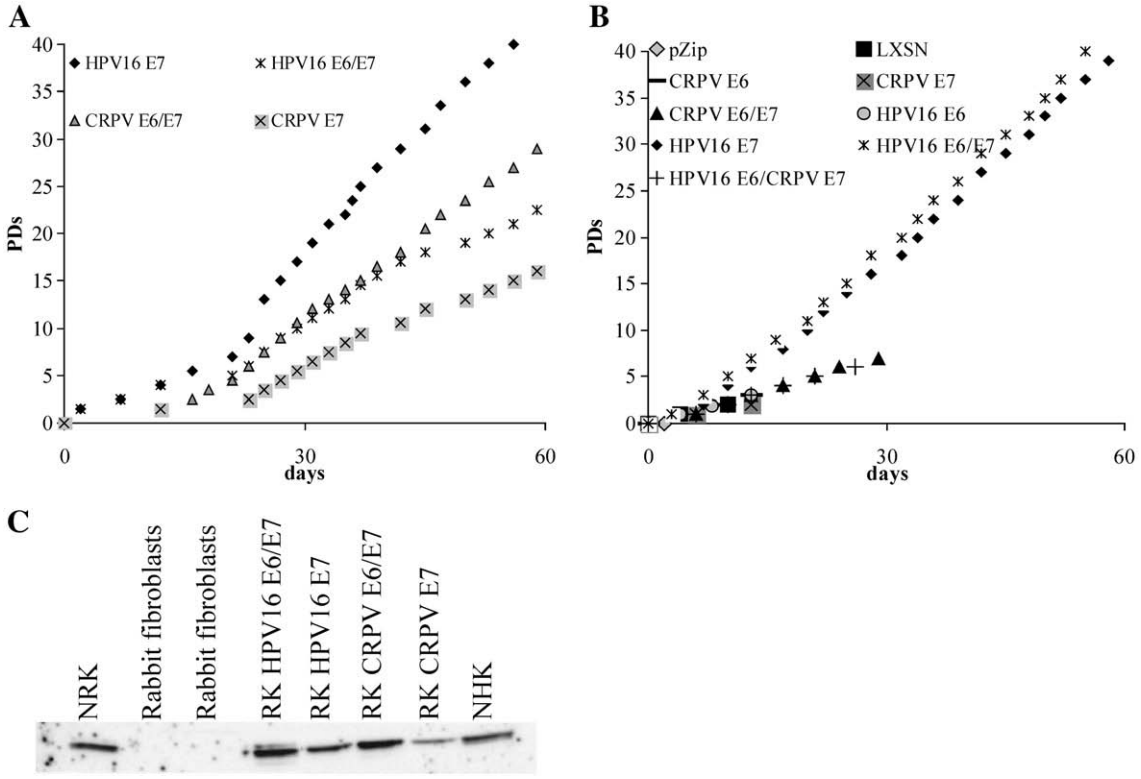

Fig. 1. (A) Long-term growth profile of immortalized rabbit keratinocytes. The number of population doublings (PDs) of the immortalized rabbit keratinocytes (RK) within 2 months is displayed. PD 0 refers to the point at which drug selection was complete. RK containing CRPV E6/E7, HPV16 E7 or HPV16 E6/E7 demonstrate an immortalized phenotype. Interestingly, RK-CRPV E7 show immortalization after a certain time span considered as a cellular crisis. Keratinocytes infected with other retroviral vectors (as pZip, pLXSN, CRPV E6 and HPV16 E6) did not survive drug selection; therefore, they are not included in this graph. (B) Long-term growth profile of human keratinocytes. In long-term cultivation, the drug-resistant human keratinocytes (HK) cell lines HK-CRPV E6/E7 and HK-HPV16 E6/CRPV E7 showed extended life span. HK with HPV16 E7 and HPV16 E6/E7 were immortalized. The other HK-lines showed similar population doublings compared to the controls, HK-pZip and HK-pLXSN. (C) Western blot analysis of cytokeratin in different keratinocytes lines. As antibody anti-Cytokeratin Type II (Cymbus Biotechnology) was used. Normal human or rabbit keratinocytes (NHK or NRK), respectively, served as positive control, rabbit fibroblasts (RF) as negative control.

no extended growth potential in comparison to NRK cells and are not displayed in the diagram. RK cell lines with CRPV E6/E7, HPV16 E7 alone or HPV16 E6/E7, however, revealed immortalized phenotypes as they continued to grow over a period of more than 6 months. To our surprise, rabbit keratinocytes containing CRPV E7 (RK-CRPV E7) alone were also immortalized but went at early passages after G418 selection through a crisis of approximately 21 days marked by very slow proliferation. After overcoming crisis, the resultant cell line, RK-CRPV E7, stabilized and became nearly as proliferative as cells that contained both CRPV E6 and E7 genes (Fig. 1A). The immortalization assays were repeated at least twice with keratinocyte populations of different donor rabbits. In order to confirm that the immortalized populations are derived from keratinocytes and not of fibroblast origin, we performed Western blotting for cytokeratin, using rabbit fibroblasts as a negative control (Fig 1C).

CRPV E7 is not capable of immortalizing human keratinocytes but seems to extend life span in combination with CRPV E6 or HPV16 E6

Normal human keratinocytes were infected with retroviruses and selected with G418 as described above, yielding the following G418-resistant human keratinocytes (HK) lines: “HK-pZip”, “HK-pLXSN”, “HK-CRPV E6”, “HK-CRPV E7”, “HK-HPV16 E6”, “HK-HPV16 E7”, “HK-HPV16 E6/E7”, “HK-CRPV E6/E7” and “HK-CRPV E7/HPV16 E6”. In long-term cultivation, the resistant cell lines HK-CRPV E6/E7 and HK-HPV16 E6/CRPV E7 demonstrated a slightly extended life span. HK containing HPV16 E7 and HPV16 E6/E7 were immortalized. The other HK lines showed similar passage number compared to human keratinocytes containing pZip or pLXSN before they stopped growing. In contrast to its immortalizing properties in rabbit keratinocytes, CRPV E7 by itself was not able to immortalize human keratinocytes but seemed to confer even retarded growth (Fig. 1B).

The presence and expression of the viral genes in the rabbit cell lines is detectable by PCR and RT-PCR

For verification of the presence of the retrovirally transduced CRPV or HPV16 E6 and E7 genes in the immortalized RK lines, PCR was performed. AVS cells, containing the complete CRPV genome and SiHa cells, containing HPV16, respectively, were used as positive controls, as shown in Fig. 2A. This analysis clearly shows the presence of the correct DNA in the respective cell lines and excludes cross contamination. To confirm the

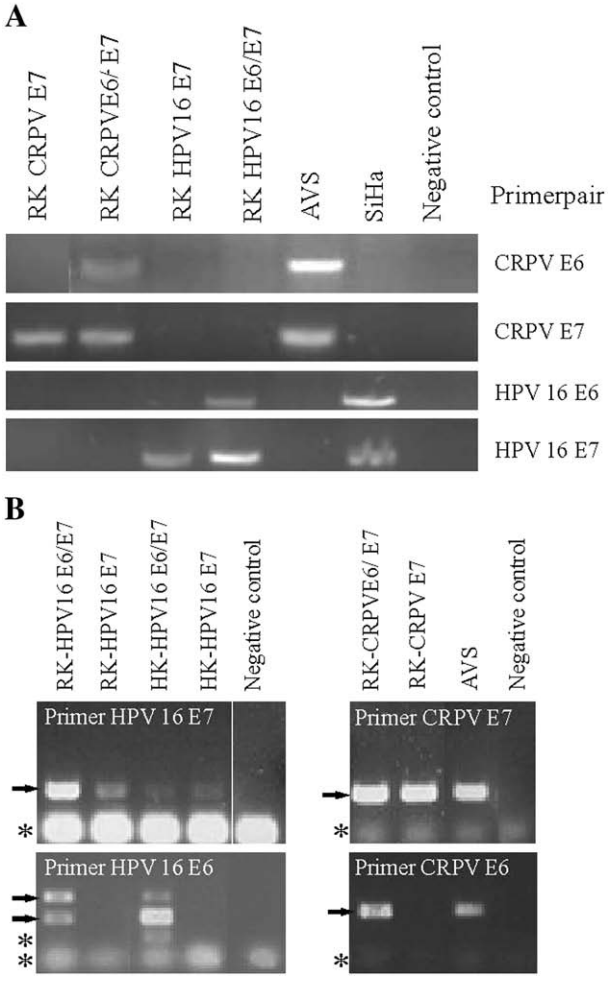

Fig. 2. (A) PCR analysis of CRPV and HPV16 E6 and E7 genes in the immortalized rabbit lines. Total cellular DNA was extracted from pelleted culture cells and PCR was performed with specific primers for CRPV and HPV16 E6 and E7. Aliquots were separated in an agarose gel and visualized by ethidium bromide staining. AVS cells, containing the complete CRPV-genome, and SiHa cells, containing HPV16, respectively, were used as positive controls. Negative control samples contained no template DNA. (B) Reverse transcription PCR analysis for E6 and E7 expression in the stable rabbit keratinocyte lines. Total RNA was isolated from the immortalized rabbit keratinocytes lines and reverse transcribed with specific primers for E6 and E7 of CRPV and HPV16, following amplification by PCR. The negative control reactions contained PCR mixture without template DNA. HK-HPV16 E6/E7 and HK-HPV16 E7 or AVS cells were used as positive control for HPV16 or CRPV, respectively. Aliquots were separated in an agarose gel and visualized by ethidium bromide staining. Left: Arrows indicate specific PCR products, stars indicate unspecific products or primers. In the case of HPV16 E6 primers, spliced and unspliced E6 are detectable.

expression of viral genes in the cell lines, we used RT-PCR as presented in Fig. 2B. Transcription of the viral genes was detectable in all cell lines. With primers for HPV16 E6, the unspliced E6/E7 mRNA and the spliced E6 mRNA were detectable. Quantitative real time PCR analysis (see Supplementary data) revealed comparable levels of E7 transcripts in rabbit or human keratinocytes expressing HPV16E6/E7 or HPV16 E7, which was not provided by the data from the solely qualitative analysis in Fig. 2B. The quantitative analysis also showed no differences in the levels of spliced and unspliced E6 transcripts in rabbit versus human cells.

The level of rabbit pRb is diminished in the rabbit cell lines

The key function of HPV16 E7 is to degrade pRb to activate the cell cycle, which also leads to the accumulation of $p14^{ARF}$ via E2F. Because we have already shown earlier that CRPV E7 is able to bind pRb (Schmitt et al., 1994), we analyzed the amount of rabbit pRb in the rabbit keratinocyte lines containing HPV16 or CRPV E7 proteins. Quantitative Western blot analysis revealed that rabbit pRb levels are strongly reduced in all RK cell lines compared to levels in normal rabbit keratinocytes as shown in Fig. 3. Untreated normal human keratinocytes (NHK) and human keratinocyte lines with HPV16 E7 served as control.

Detection of increased amounts of the cell cycle proteins $p19^{ARF}$, mdm2, p53 and p21 in the rabbit keratinocyte lines

To investigate alterations in the pRb-p53 pathway in the immortalized rabbit cell lines, we analyzed protein levels of rabbit homologs of $p19^{ARF}$, mdm2, p53 and p21. Western blot analysis demonstrated in all immortalized cell lines increased

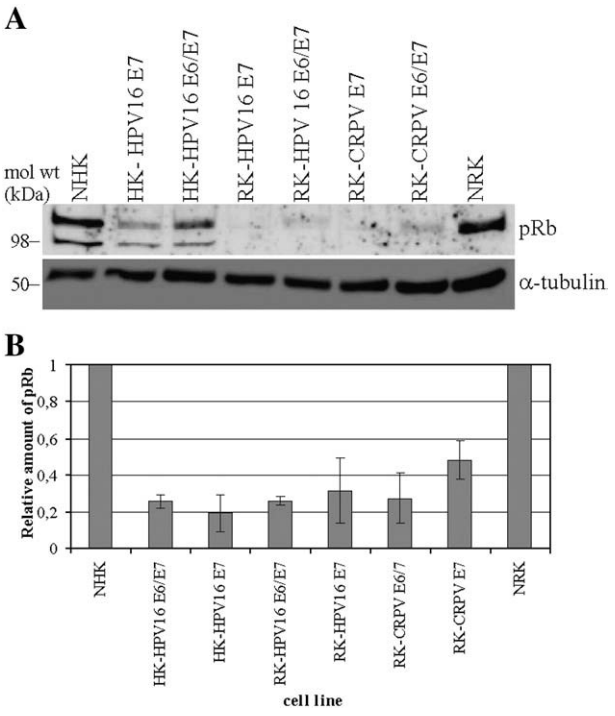

Fig. 3. Quantitative analysis of pRb levels in immortalized rabbit keratinocytes. Western blots of pRb (M-135, Santa Cruz) were performed and pRb and α-tubulin signals were quantified as described in Materials and methods. Normal human keratinocytes and HPV16 E7 expressing human keratinocytes served as positive control. The human pRb and rabbit pRb signals were corrected for the respective α-tubulin signals and are presented relative to the signals obtained with normal human keratinocytes (NHK) or normal rabbit keratinocytes (NRK), respectively, which were set to 1. (A) Representative Western blot including α-tubulin as loading control. Molecular weight (mol wt) in kilodalton is presented on the left. (B) Average relative amount of pRb. The columns represent the average of at least three independent Western blots; the bars show the standard deviation.

levels of rabbit p19^{ARF}, mdm2, p53 and p21 as compared to normal rabbit keratinocytes as control (Fig. 4).

None of the CRPV E6 proteins is able to degrade human or rabbit p53 in vitro

As previously shown, neither long nor short E6 of CRPV were capable of binding p53 from F9 mouse embryonal carcinoma cells (Harry and Wettstein, 1996). Thus, we wanted to verify these data for rabbit p53 using *in vitro* degradation assays. As shown in Fig. 5A, HPV16, HPV18 E6 or CRPV E6 is not able to degrade rabbit p53 *in vitro*. Human p53 is degraded efficiently by E6 of HPV16 and HPV18, but not by CRPV E6.

CRPV E6 is in contrast to high-risk E6 unable to bind rabbit E6AP

The main feature of high-risk HPV E6 proteins is their ability to recruit the E3-ubiquitin ligase E6AP and alter its substrate specificity towards p53. Because our data showed that CRPV, HPV16 and HPV18 E6 failed to degrade rabbit p53, we analyzed in MBP pull-down assays whether this is due to the inability to interact with rabbit E6AP. MBP fusion proteins of CRPV LE6, SE6, HPV16 or HPV18 E6 were incubated with cell extracts from RK-CRPV E7 and tested for interaction with rabbit E6AP and rabbit p53. Extracts from HEK293 cells incubated with HPV16 and HPV18 E6 fusion proteins served as positive control. As shown in Fig. 5B, HPV16 and HPV18 E6 were able to bind both rabbit and human E6AP, whereas CRPV LE6 and SE6 revealed no affinity to rabbit E6AP. Confirming our data from the *in vitro* degradation assay, rabbit p53 showed no interaction with HPV16 or HPV18 E6, whereas human p53 did. None of the CRPV E6 proteins were able to retain rabbit p53 on the column.

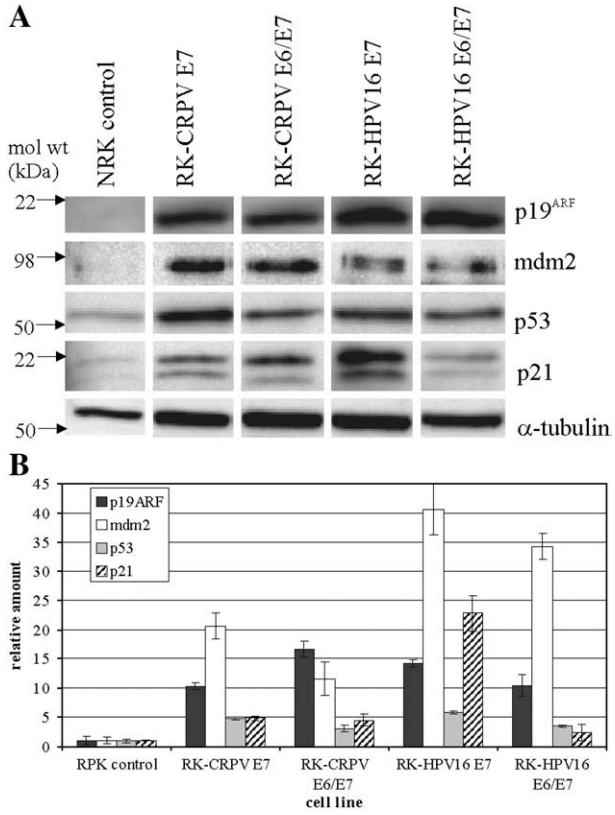

Fig. 4. Protein levels of the p19^{ARF}/p53 pathway proteins in rabbit keratinocytes. Lysates of the immortalized rabbit keratinocytes (RK) were analyzed by SDS–PAGE and immunoblotting for p19^{ARF} (14P03/ DCS-241, Neomarkers), mdm2 (SMP14, Santa Cruz), p53 (DO-1, Santa Cruz) and p21 (SX-118, Pharmingen) proteins. Measurements were repeated several times; here representative Western blot stainings are shown for each protein including α-tubulin (Ab-1, Calbiochem) as a loading control. On the left, the molecular weight (mol wt) in kilodalton is displayed.

Inhibition of the proteasome does not influence the rabbit p53 levels in rabbit keratinocytes

Inhibition of proteasomal activity with MG-132 in HeLa cells resulted in an accumulation of p53 compared to control cells. In contrast, no significant change of rabbit p53 amount was detected in rabbit keratinocytes containing CRPV E7 or CRPV E6/E7, as shown in Fig. 6.

Induction of rabbit p53 by treatment with mitomycin C

To analyze if rabbit p53 is still inducible and transcriptionally active by DNA damage, we treated the rabbit keratinocyte lines with mitomycin C (MMC), which causes damage of DNA by alkylation and cross-linking and thereby p53 accumulation (Abbas et al., 2002; Hess et al., 1994). Normal rabbit keratinocytes (NRK) and normal human keratinocytes (NHK) served as controls. In Fig. 7, we demonstrate that MMC treatment caused stabilization of rabbit or human p53 in all treated cell lines, which was accompanied by increased cell death of the treated versus non-treated cells. Subsequent rabbit or human p21 accumulation was detected in the MMC-treated immortalized rabbit keratinocyte cell lines and NHK. P21 showed no detectable levels in normal rabbit keratinocytes.

Rabbit p53 is localized mainly in the nucleus

P53 signaling can be influenced by altering the subcellular localization, for example, by nuclear exclusion. To investigate the phenomenon of increased rabbit p53 expression in unaffectedly proliferating rabbit keratinocyte lines, we analyzed rabbit p53 localization by immunofluorescence staining. As shown in Fig. 8, rabbit p53 localized strictly to the nuclei in all cell lines, except for RK-CRPV E6/E7 that revealed in addition to the nuclear staining a distinct rabbit p53 signal in the cytoplasm.

Discussion

To investigate the immortalizing properties of the cottontail rabbit papillomavirus (CRPV), we established human and for the first time rabbit keratinocyte lines stably expressing CRPV or HPV16 E6 and E7 together or separately after retroviral gene transfer.

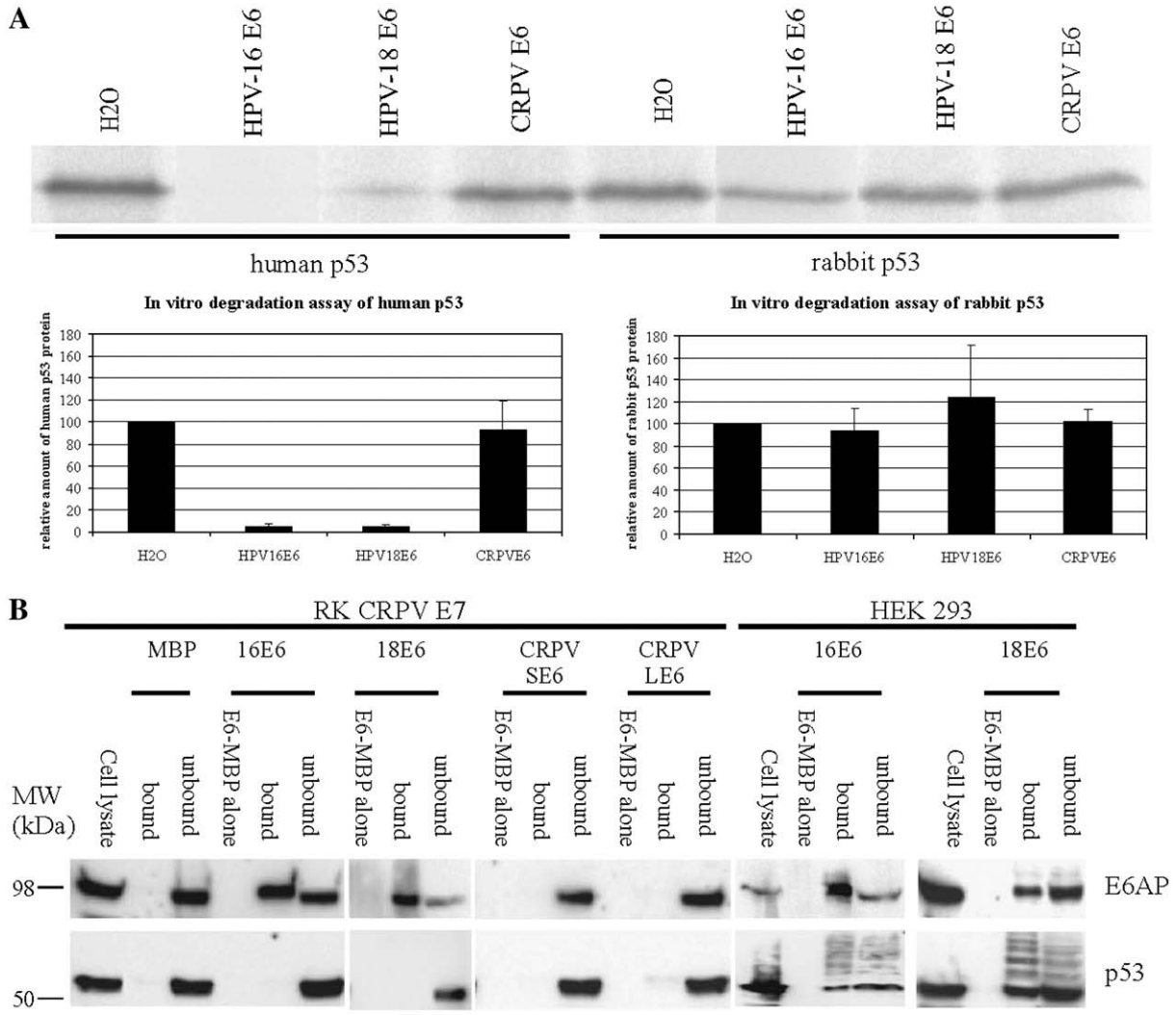

Fig. 5. *In vitro* biological properties of the E6 proteins of CRPV, HPV16 and HPV18. (A) E6-mediated degradation of human and rabbit p53 *in vitro*. *In vitro* translated p53 was incubated with *in vitro* translated E6 protein of CRPV, HPV16 and HPV18 or water-primed lysate (H_2O) for 2 h. On top of the figure, an autoradiogram of a representative degradation assay is displayed. At the bottom, the average relative amounts of p53 remaining after 2 h of incubation quantified by phosphoimaging are shown. Data are relative to the water-primed control, which was set to 100%. The columns represent the average of at least three independent experiments, the bars the standard deviation. (B) *In vitro* ability of E6 proteins of CRPV, HPV16 and HPV18 E6 to interact with rabbit p53 and E6AP in MBP pull-down assays. RK-CRPV E7 and HEK293 cells were lysed and incubated with immobilized MBP fusion proteins of CRPV short and long E6 as well as HPV16 or HPV18 E6. Beads were washed and MBP-E6 and interacting proteins were eluted via maltose. Proteins were analyzed by SDS–PAGE and Western blotting using anti-p53 (DO-1, Santa Cruz) and anti-E6AP (H-182, Santa Cruz) antibodies. On the left, the molecular weight (in kDa) in kilodalton is displayed.

Rabbit keratinocytes (RK) were immortalized by CRPV E7 itself after overcoming cellular crisis or by CRPV E6 and E7 together without crisis. Interestingly, CRPV E7 by itself was not able to alter life span of human keratinocytes. Thus, CRPV E7 showed species-specific differences with regard to its immortalizing abilities in human and rabbit keratinocytes. However, expression of CRPV E6/E7 or a combination of CRPV E7 and HPV16 E6 in human keratinocytes caused only a slightly prolonged life span, in contrast to the HPV16 E7- or HPV16 E6/E7-mediated immortalization of human keratinocytes that has been described before (Halbert et al., 1991; Hawley-Nelson et al., 1989; Munger et al., 1989). Interestingly, HPV16 E7 and HPV16 E6/E7 were able to overcome the species barrier and to immortalize rabbit keratinocytes, underlining their properties as potent oncogenes.

Because CRPV E7 is sufficient to immortalize normal rabbit keratinocytes by itself, the situation is comparable to the HPV16 E7-mediated immortalization of human keratinocytes (Hawley-Nelson et al., 1989; Munger et al., 1989). Further CRPV E6 expressed together with E7 omitted the cellular crisis observed with E7 only and therefore cooperated in the immortalization process like HPV16 E6 as reported previously (Halbert et al., 1991). However, it must be taken into account that HPV16 E6 is able to degrade p53, whereas CRPV E6 is neither capable of degrading human or rabbit p53 nor capable of interacting with E6AP, as we have shown here. CRPV E6 alone did not exhibit immortalizing abilities in rabbit or human keratinocytes but revealed transforming activities in fibroblast lines as shown earlier (Meyers et al., 1992; Schmitt et al., 1994). Thus, the mechanism how the E6 proteins of CRPV do accelerate the

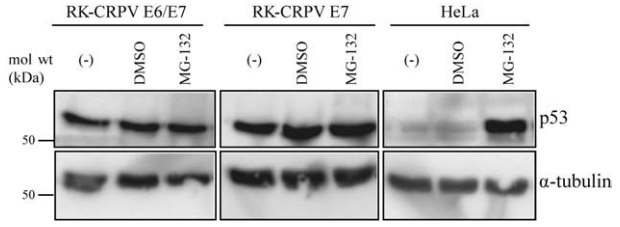

Fig. 6. Analysis of p53 after proteasomal inhibition with MG-132. RK-CRPV E6/E7, RK-CRPV E7 or HeLa cells were incubated with 0.1% DMSO or 10 μM of the proteasome inhibitor MG-132 solved in 0.1% DMSO for 6 h for RK and 4 h for HeLa cells. Untreated cells are marked by (−). Cells were lysed and rabbit or human p53 (DO-1, Santa Cruz) and α-tubulin as a loading control were detected by Western blotting. One representative Western blot analysis is shown here. The molecular weight (mol wt) in kilodalton is displayed on the left.

immortalization of RK-CRPV E6/E7 is particularly interesting. In contrast to HPV E6 proteins, there is very little known about CRPV E6's cellular interaction partners. CRPV E6 could possibly affect telomerase activity, as reported for HPV16 E6 (Mantovani and Banks, 2001), a property that is independent of the ability to degrade p53 and is not sufficient for immortalization of cells (Klingelhutz et al., 1996). However, CRPV E6's deficiency to bind E6AP makes this highly unlikely (Gewin et al., 2004). Unfortunately, with the currently available rabbit genomic data, we failed to detect a rabbit mRNA homologous to any known TERT cDNA and could not test for rabbit telomerase induction (data not shown). The influence of CRPV E6 on Bak, a proapoptotic protein, which is targeted by genital and cutaneous HPVs (Jackson et al., 2000; Thomas and Banks, 1999), could not be analyzed because the commercially available antibodies were not functional for detection of the rabbit homolog. Recently, an association of CRPV E6 proteins with the hDLg/SAP97 tumor suppressor protein was demonstrated. High-risk HPV E6 proteins bind and target hDlg for degradation, whereas CRPV E6 did not lead to degradation of hDlg (Du et al., 2005). This finding can be confirmed by our data showing the inability of CRPV E6 to bind the ubiquitin ligase E6AP, an interaction that seems to be necessary for degradation of hDlg (Kuballa et al., 2007). Further investigations are necessary to clarify the mechanism of CRPV E6's ability to cooperate with E7 in immortalization of rabbit keratinocytes.

CRPV E7 seems to be the major immortalizing factor in rabbit keratinocytes and thus comparable to high-risk HPV E7 proteins. A main feature of HPV16 E7 in carcinogenesis is its ability to associate with human pRb and promote its degradation via the proteasome pathway (Munger et al., 2001). The 14-kDa CRPV E7 protein shares some biological properties with HPV16 E7: both bind human pRb at the same C-terminal domain and disrupt the complex between pRb and the transcription factor E2F. CRPV E7 shows 11–12% relative binding to human pRb compared to HPV16 E7 (Defeo-Jones et al., 1993; Haskell et al., 1993; Schmitt et al., 1994). Here we demonstrate that both CRPV and HPV16 E7 were able to reduce rabbit pRb levels in rabbit keratinocytes. Treatment of the CRPV E7 containing rabbit keratinocytes with the proteasome inhibitor MG-132 led to enhanced amounts of pRb, referring to a mechanism for pRb reduction linked to the proteasomal degradation machinery (data not shown). Taken together, this supports the assumption that CRPV E7 is using an inactivation mechanism of rabbit pRb comparable to the mechanism of HPV16 E7 and that this ability of CRPV E7 could explain its competence to immortalize rabbit cells. However, there might be additional events necessary taking into account the cellular crisis before rabbit keratinocytes become immortalized.

Rabbit keratinocytes immortalized by the CRPV or HPV16 E7 and E6/E7 genes demonstrate a steep rise of rabbit $p19^{ARF}$, probably due to the degradation of rabbit pRb by E7 and subsequent release of E2F transcription factors as shown for the murine/human system before (Bates et al., 1998; Komori et al., 2005). According to the model, this will cause inhibition of rabbit mdm2 and accumulation of rabbit p53 (Honda and Yasuda, 1999; Zhang et al., 1998), an instance which could be demonstrated here in all immortalized rabbit keratinocytes. These findings suggest a pRb pathway operating in rabbit cells similarly to the human system and stress the resemblance of the E7 of CRPV and HPV16. However, in the case of HPV16 E7, it has been reported that p53 stabilization is independent of p19ARF (Seavey et al., 1999), as shown in mouse embryo fibroblasts lacking $p19^{ARF}$. We cannot rule out this point completely for the rabbit keratinocytes in our experiments.

In order to further enlighten the p53 stabilization in the rabbit keratinocytes, we analyzed the interactions between p53, E6AP

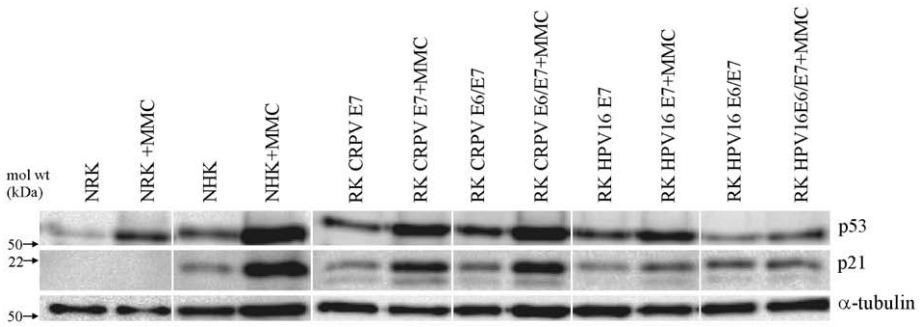

Fig. 7. Levels of rabbit p53 and p21 protein after mitomycin C treatment of NRK, NHK and immortalized rabbit keratinocytes. Cells were treated with 7 μg/ml mitomycin C (MMC) in K-SFM for 14 h (designated as "+MMC") and lysed afterwards. Negative controls received K-SFM only. Cell lysates were analyzed by SDS–PAGE and immunoblotting for p53 (DO-1, Santa Cruz) and p21 (SX-118, Pharmingen). The measurement was repeated at least twice; here one representative Western blot is shown including the loading control α-tubulin. Molecular weight (mol wt) in kilodalton is displayed on the left.

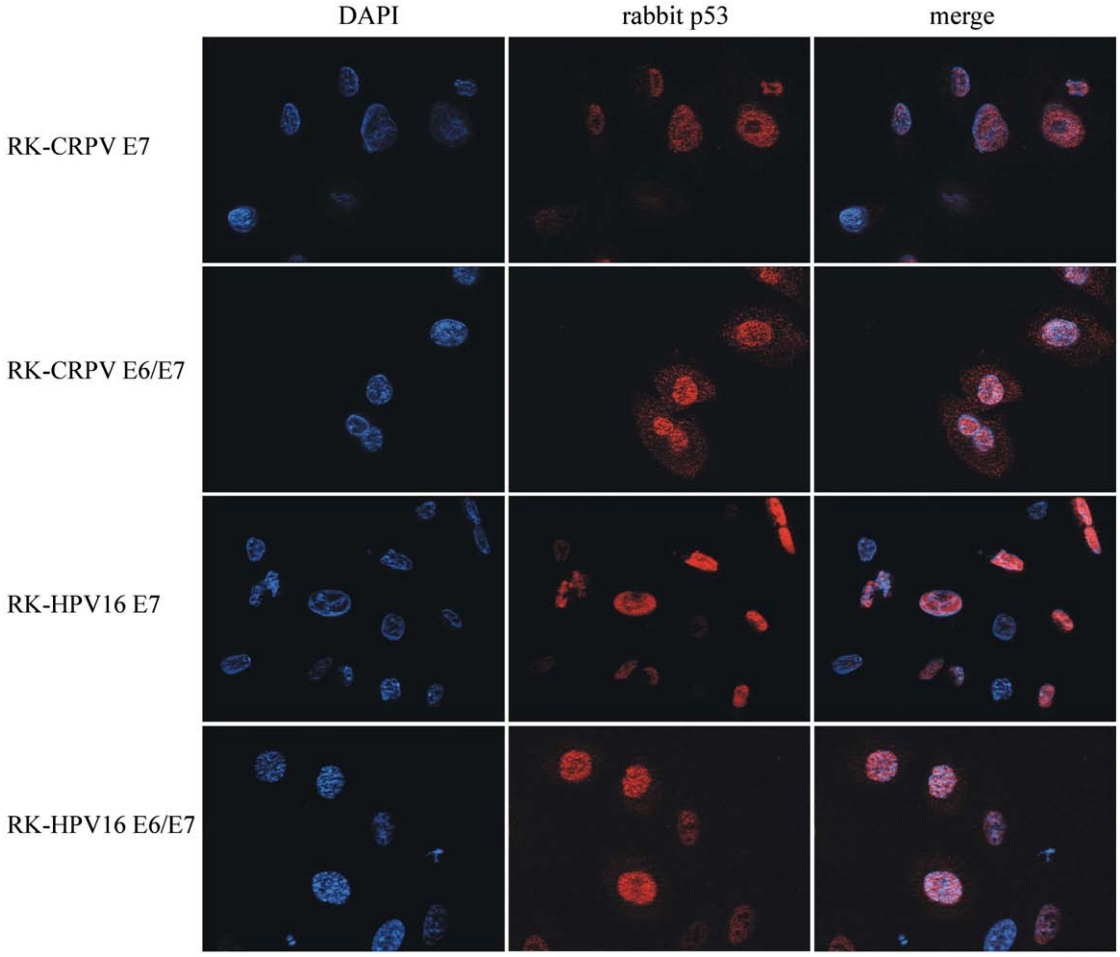

Fig. 8. Immunofluorescence analysis of p53 in rabbit keratinocyte lines. The localization of p53 in the different rabbit cell lines was evaluated by staining of nuclei by 4′,6-diamidino-2-phenylindole (DAPI) and immunostaining of rabbit p53 (DO-1, Santa Cruz) and a Cy3-labeled secondary antibody. The third column shows the merge of the first two columns.

and the E6 proteins of CRPV and HPV18. As mentioned above, none of the CRPV E6 proteins were able to degrade rabbit or human p53. Interestingly, HPV18 E6 was neither able to bind nor to degrade rabbit p53 *in vitro*. The inability of HPV18 E6 to interact with rabbit p53 might be due to species-specific structural differences as it is capable of efficiently binding and degrading human p53. An alignment of human and rabbit p53 showed 85% conservation on the amino acid level (data not shown). In addition, the CRPV E6 proteins were unable to interact with rabbit E6AP, whereas HPV18 E6 was able to bind rabbit E6AP. These results indicate that the complex formation of E6, E6AP and p53, a common feature for high-risk HPV E6 (Scheffner et al., 1993), does not occur in rabbit keratinocytes. Obviously, the transcriptional modulation functions that have been described for the human E6–E6AP complex (Kelley et al., 2005) are dispensable for tumor formation in the rabbit.

Hence, the question arises, how a p53-mediated growth arrest is abrogated. This situation is comparable to the setting in HPV16 E7 expressing human cells, which display stabilized p53 resulting from an extension of its half-life, but uncompromised growth abilities (Demers et al., 1994; Eichten et al., 2002; Jones et al., 1997; Jones and Munger, 1997). In this case, it has been demonstrated that the stabilized p53 in HPV16 E7 expressing cells is transcriptionally inert (Eichten et al., 2002) and that p21 expression is increased by protein stabilization rather than through p53-mediated transcriptional induction (Jian et al., 1998; Jones et al., 1999).

As HPV16 E7 can interact with the S4 subunit of the 26S proteasome (Berezutskaya and Bagchi, 1997), we studied whether p53 might be stabilized in the immortalized rabbit keratinocytes by interference with the proteasomal degradation system. We could demonstrate that the p53 level in both CRPV E7 and CRPV E6/E7 containing RK cells was not influenced by inhibition of the proteasome with MG-132. This indicates that probably rabbit mdm2 is blocked by $p19^{ARF}$ and mainly responsible for rabbit p53 degradation, as there is no further increase of rabbit p53 after proteasomal inhibition.

In contrast, we could demonstrate by treatment of the rabbit cell lines with mitomycin C (MMC) that the stabilized rabbit p53 is inducible by DNA damage signaling. Interestingly, rabbit p21 levels also increased in response to MMC treatment implying an intact p53 transactivation.

Another possibility of interfering with p53 functions is the mutations of p53 occurring within the process of immortalization. It has been reported that p53 is often mutated in skin tumors (Ananthaswamy and Pierceall, 1992). However, because

we could show that rabbit p53 is still inducible by DNA damage this suggests a wild-type p53 transactivation function. Furthermore, exclusively wild-type rabbit p53 sequence was found in CRPV containing carcinomas and papillomas (S. Tkaczik, unpublished data).

In addition we tested whether rabbit p53 is functionally inactivated by nuclear exclusion. This has been reported for different carcinomas (Bosari et al., 1995; Moll et al., 1992; Schlamp et al., 1997) or for viral oncoproteins, which are able to mislocalize p53 to the cytoplasm (Elmore et al., 1997; van den Heuvel et al., 1993). Here we show by immunofluorescence analysis that in the immortalized rabbit keratinocytes no dramatic shift in the subcellular localization was observed for rabbit p53, but CRPV E6 seems to affect a minor amount of total rabbit p53 with respect to its nuclear localization. This finding might give hints to additional CRPV E6 functions. However, the exact mechanism, how CRPV E7 does abolish p53-mediated growth arrest or apoptosis, remains unclear.

Taken together, CRPV E7 was able to immortalize normal rabbit keratinocytes by itself after going through a crisis. The immortalization by CRPV E7 was enhanced by co-expression of CRPV E6 preventing cellular crisis. The immortalized rabbit cells showed high proliferation but contained elevated levels of rabbit p53, probably due to $p19^{ARF}$-mediated mdm2 inhibition caused by degradation of rabbit pRb via CRPV E7. Rabbit p53 was still inducible by DNA damage and displayed wild-type functions. Our findings validate the rabbit animal model by showing immortalizing abilities of CRPV E7 in rabbit keratinocytes thereby stressing the role of papillomaviruses in cutaneous carcinogenesis. The established cell culture system complements the animal system giving the possibility to evaluate *in vitro* properties of CRPV and thus provides a valuable tool for the further investigation of mechanisms leading to skin cancer.

Materials and methods

Cell culture

Primary human foreskin keratinocytes designated as NHK (normal human keratinocytes) were isolated from neonatal foreskins as described (Ruesch et al., 1998) and cultivated in complete keratinocyte serum-free medium (K-SFM) (Invitrogen, Karlsruhe, Germany) supplemented with gentamicin (0.5 mg/mL). Primary rabbit keratinocytes were isolated from New Zealand White rabbit skin as followed: Approximately 2×3 cm sized rabbit skin pieces were washed several times in PBS, incubated with 10 ml dispase (2.4 U/l) (Roche) for 4 h at 37 °C. After disconnection from the subcutis, the epidermis was incubated for 10 min with trypsin (0.25% trypsin, 1 mM EDTA) (Invitrogen) followed by mechanical disruption, inactivation of trypsin and purification by using a cell strainer (Falcon, Becton Dickinson, Heidelberg, Germany). After centrifugation, cells were plated on Primaria culture dishes (Falcon) and designated as NRK (normal rabbit keratinocytes). Primary cultures, resulting cell lines and AVS cells (rabbit keratinocytes containing the complete CRPV genome; Huber et al., 2004), were maintained in complete K-SFM without antibiotics. Cells were used for retroviral infection within a few days after isolation. SiHa, HeLa, HEK293 and Phoenix cells were maintained in D-MEM (Invitrogen) with 10% of bovine calf serum. All cells were cultivated at 37 °C and 5% CO_2 in humidified atmosphere.

Generation of amphotropic retroviruses

High-titered amphotropic recombinant retroviruses were generated by transient transfection of retroviral vectors into Phoenix cells (ATCC # SD 3443) using the calcium phosphate precipitation method. The following plasmids were used: pZipNeo SV(X)-1 (Cepko et al., 1984), pZipNeoCRPVE6, pZipNeoCRPVE7 (Meyers et al., 1992), pLXSN (Clontech, Heidelberg, Germany), pLXSNHPV16E6, pLXSNHPV16E7 and pLXSNHPV16E6/E7 (Halbert et al., 1991).

Retroviral infection of normal keratinocytes

Forty-eight hours after transfection of Phoenix cells, the cell culture supernatants were filtered through 0.45-μm sterile filters, and 2 ml aliquots was mixed with 4 ml of K-SFM and 4 μg/ml polybrene (Sigma). The mixture was incubated for 8 h with NHK or NRK cells grown on 100-mm-diameter dishes to 70% confluency. Then G418 selection (50 μg/ml) was applied for 10 days. Coinfection was carried out by using two different supernatants simultaneously. Stable G418-resistant cell lines were maintained in supplemented K-SFM and long-term growth was monitored. Rabbit keratinocytes (RK) lines were designated as RK-CRPV E7 (pZipCRPVE7), RK-CRPV E6/E7 (coinfection with pZipCRPVE6 and pZipCRPVE7), RK-HPV16 E7 (pLXSNHPV16E7) and RK-HPV16 E6/E7 (pLXSNHPV16E6/E7), human keratinocytes (HK) lines as "HK-pZip" (pZipNeo), "HK-pLXSN" (pLXSN), "HK-CRPV E6" (pZipCRPVE6), "HK-CRPV E7" (pZipCRPVE7), "HK-HPV16 E6" (pLXSNHPV16E6), "HK-HPV16 E7" (pLXSNHPV16E7), "HK-HPV16 E6/E7" (pLXSNHPV16E6/E7), "HK-CRPV E6/E7" (coinfection with pZipCRPVE6 and pZipCRPVE7) and "HK-CRPV E7/HPV16 E6" (coinfection with pZipCRPVE7 and pLXSNHPV16E6).

Long-term growth analysis of stable cell lines

To determine the number of population doublings (PDs) of the stable human or rabbit keratinocytes over a certain time period, we grew the cells on 100-mm-diameter dishes and trypsinized when they reached approximately 80% confluence. PDs were calculated taking into consideration the number of passages and split ratio.

Drug treatments of culture cells

Rabbit keratinocyte lines were treated with 7 μg/ml mitomycin C (MMC) (medac, Wedel, Germany) in K-SFM at a confluence of about 70% for 14 h. Control cells received fresh culture medium without MMC.

For inhibition of proteasomal protein degradation, approximately 1×10^7 HeLa or 3×10^6 RK-CRPV E7 and RK-CRPV E6/E7 cells, respectively, were incubated with 0.1% DMSO or 0.1% DMSO and 10 μM proteasome inhibitor MG-132 (Calbiochem, Merck-Biosciences, Darmstadt, Germany). Incubation time was 6 h for RK and 4 h for HeLa cells, followed by trypsinization, lysis in NP40 lysis buffer (130 mM NaCl, 50 mM Tris pH 8.0, 1 mM EDTA, 1% NP-40, 1 mM DTT) and analysis by quantitative Western blotting.

PCR

Pelleted cultured cells (2×10^6) were incubated with proteinase K followed by extraction of total DNA using the Biorobot EZ-1 and the EZ-1 DNA Tissue kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. PCR was performed with 200 ng of cellular DNA and 40 pmol of oligonucleotides using AmpliTaq Gold DNA Polymerase (Applied Biosystems, Darmstadt, Germany) under following PCR conditions: 5 min 95 °C; 40 cycles with 1 min 95 °C, 30 s 55 °C, 60 s 72 °C; 10 min 72 °C. The primer sequences were as follows: CRPV E6: 155 forward TGGAGAACTGCCTGCCACG, 693 reverse TTCCTCCAGATCCACCGGAG; CRPV E7: 1081 forward GGCAGAACTCCTAAGCTTAGTGAGC, 1359 reverse TCAGTTACAACACTCCGGGCA; HPV16 E6: 140 forward CCACAGTTATGCACAGAGCT, 557 reverse ACAGCTGGGTTTCTCTACGTGTTC; HPV16 E7: 563 forward TGCATGGAGATACACCTACATTGC, 853 reverse GTTTCTGAGAACAGATGGGGCA.

Reverse transcription PCR

Total RNA was isolated from cultured cells with the RNeasy-Kit (Qiagen) according to the manufacturer's instructions including on-column DNase-treatment. Reverse transcription was performed using the Superscript II Reverse Transcriptase (Invitrogen), random hexamer oligonucleotides and 2 μg of total RNA as recommended by the manufacturer. cDNA was amplified using the GoTaq-DNA-Polymerase (Promega, Mannheim, Germany) according to manufacturer's instructions and the following primer pairs: CRPV E6 446 forward GCGTTGTACAGTTTGCGGA and 800 reverse ACCCCCCTCCTCGTCTTCTTCCC; HPV16 E6 101 forward GCAATGTTTCAGGACCCACAG and 557 reverse ACAGCTGGGTTTCTCTACGTGTTC; further CRPV E7 1081 forward and 1359 reverse and HPV16 E7 563 forward and 853 reverse as described above.

Western blot analysis

Total cell extracts were prepared with M-PER mammalian protein extraction reagent (PerbioScience, Bonn, Germany) according to the manufacturer's instructions. Protein concentrations of the extracts were determined by the Micro BCA Protein Assay Reagent Kit (PerbioScience). Probes with equal protein concentrations were run on SDS–polyacrylamide gels, transferred to nitrocellulose, blocked and incubated afterwards with the primary antibody overnight. The following antibodies were used: mdm2 (SMP14, mouse monoclonal, Santa Cruz Biotechnology, Heidelberg Germany), $P14^{ARF}$/p16β Ab-3 (Clone 14P03/ DCS-241, mouse monoclonal, Neomarkers), p21 (SX-118, mouse monoclonal, Pharmingen BD-Biosciences, Heidelberg, Germany), p53 (DO-1, mouse monoclonal, Santa Cruz), α-Tubulin (Ab-1, mouse monoclonal, Calbiochem), pRb (M-135, rabbit polyclonal, Santa Cruz), Cytokeratin Type II (mouse monoclonal, Cymbus Biotechnology) and E6AP (H-182, rabbit polyclonal, Santa Cruz). The respective secondary antibodies were conjugated to horseradish peroxidase. Blots were developed with SuperSignal West Femto (PerbioScience) as a substrate and visualized by the Fluor-S Max MultiImager (Bio-Rad, München, Germany). Band intensities were quantified using the Quantity One Quantitation software package (version 4) (Bio-Rad) and signals were normalized against the respective levels of α-tubulin to correct for unequal loading of the gels.

In vitro degradation assays

Plasmids Rc/CMV-p53, pcDNA3.1D-16 E6 and pcDNA3.1D-18 E6 were previously described (Hiller et al., 2006). The CRPV E6 gene was amplified by PCR using the cloned CRPV genome as a template and cloned into pSG5 and verified by sequencing. Rc/CMV-p53 or pSG5/pcDNA3.1-E6 plasmids were transcribed and translated separately *in vitro* using the TNT coupled rabbit reticulocyte lysate system (Promega) in the presence of [^{35}S]-methionine (>1000 Ci/mmol) or [^{35}S]-cysteine (>1000 Ci/mmol) (Amersham, Munich, Germany), respectively, according to the manufacturer's instructions. An aliquot of each translation reaction was separated by SDS–PAGE and exposed to image screens, and the amount of labeled proteins was then quantified by using a BAS-1800 PhosphoImager (Fujifilm) and the AIDA software version 2.0 (Raytest, Straubenhardt, Germany). After correcting for the different amounts of cysteines or methionines, respectively, normalized amounts of E6 proteins were mixed with p53 at a ratio of 1:11. Volumes were adjusted using water-primed lysate. Reaction mixtures were incubated at 30 °C for 2 h. The residual proteins were resolved by SDS–PAGE and visualized as described above.

Maltose binding protein pull-down assays

HPV16 E6, HPV18 E6 and CRPV LE6 and SE6 were cloned into the pMal-c2x plasmid (NEB, Frankfurt, Germany) yielding pMal-c2X-HPV16E6, pMal-c2X-HPV18E6, pMAL-c2X-CRPVLE6 and pMAL-c2X-CRPVSE6. Transformed *Escherichia coli* BL21 CDE3 Rosetta2 bacteria were grown to an OD600 of 0.6, cells were shifted to 20°C and then 0.5 mM IPTG was added for 1 h. Cells were harvested by centrifugation; pellets were resuspended in 1 ml of TMN-buffer (50 mM Tris pH 8.0, 12.5 mM $MgCl_2$, 0.1% NP-40, 0.1% DTT) and then disrupted by ultrasonication (Bandelin Sonoplast UW2200) on ice. Cleared supernatants were incubated with 50 μl of amylose agarose beads (NEB) for 1 h at 4 °C and then extensively washed with TMN buffer. RK-CRPV E7 and HEK293 cells were harvested by trypsinization and lysed in NP40 lysis buffer (130 mM NaCl, 50 mM Tris pH 8.0, 1 mM EDTA, 1% NP-40, 1 mM DTT). Cell

lysates equivalent to 3×10^7 cells were incubated with immobilized maltose binding protein (MBP) fusion proteins for 2 h at 4 °C. Subsequently, the resin was washed several times with TMN buffer and the retained proteins were eluted with TMN buffer containing 20 mM maltose and analyzed by Western blotting.

Immunofluorescence microscopy

For indirect immunofluorescence microscopy analysis, cells were grown on MatTek glass bottom culture dishes (MatTek Corp., Ashland, MA), fixed in 4% paraformaldehyde for 10 min and permeabilized with 0.2% Triton-X. Staining was performed with p53 antibody (DO-1, Santa Cruz) diluted 1:100 in PBS/3% bovine serum albumin or appropriate controls for 1 h at room temperature. Cells were subsequently washed four times with PBS and incubated with Cy3-conjugated anti-mouse IgG antibodies diluted 1:300 in PBS/3% bovine serum albumin. Unbound antibody was removed by extensive washing with PBS; 4′,6-diamidino-2-phenylindole (DAPI) in PBS was briefly added to stain for DNA and then unbound DAPI was removed by washing with PBS. Fluorescence signals were visualized with a Zeiss Axiovert 200 M microscope featuring the ApoTome technique and the respective fluorescence filter sets for DAPI and Cy3. Pictures were taken with an AxioCam MRm camera and processed with AxioVision software version 4.3 (Carl Zeiss AG, Oberkochen, Germany).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2007.11.006.

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